REMARKS

Claims 3-5 and 11-14 are pending in the subject application. Claim 3 has been amended to more particularly point out that which the applicants regard as the invention. Support for amended claim 3 can be found in the specification at, *inter alia*, page 31, lines 20-22. Accordingly, upon entry of this Amendment, claims 3-5 and 11-14 will still be pending and under examination.

The Claimed Invention

Claim 3, and dependent claims 4, 5 and 11-14, provide a method for preventing exaggerated restenosis in a diabetic subject at risk of developing exaggerated restenosis which comprises administering to the subject a therapeutically effective amount of human, mouse or rat soluble receptor for advanced glycation endproducts (sRAGE) so as to prevent exaggerated restenosis in the subject.

Rejection under 35 U.S.C. §112, First Paragraph and the October 17, 2005 Examiner's Interview

In the Final Office Action, and as maintained in the Advisory Action, the Examiner rejected claims 3-5 and 11-14 under 35 U.S.C. §112, first paragraph, because the specification allegedly fails to provide adequate evidence showing that soluble RAGE from human or bovine origin, or other species, would be expected to have the same biological function as mouse sRAGE.

During the October 17, 2005 telephonic interview, the undersigned attorney, Alan J. Morrison, and Examiner Chen discussed this

rejection. Applicants wish to thank Examiner Chen for his time and consideration. During the interview, Examiner Chen indicated that this rejection could be overcome were applicants to (i) amend claim 3 to limit sRAGE to that of human or mouse origin and (ii) submit *in vivo* or *in vitro* evidence persuasively showing functional homology between mouse and human sRAGE.

In response to the Examiner's rejection and remarks during the interview, applicants first note that claim 3, as amended herein, provides a method for preventing exaggerated restenosis in a diabetic subject at risk of developing exaggerated restenosis which comprises administering to the subject a therapeutically effective amount of a *human or mouse* soluble receptor for advanced glycation endproducts (sRAGE) so as to prevent exaggerated restenosis in the subject.

Second, in support of their position that human sRAGE would be reasonably expected to have functional homology to mouse sRAGE, applicants attach as **Exhibit A** a copy of Renard, et al. ("The Human and Rat Recombinant Receptors for Advanced Glycation End Products Have a High Degree of Homology but Different Pharmacokinetic Properties in Rats," J. Pharmacology and Experimental Therapeutics, 250(3): 1458-1466 (1999)).

Renard, et al., teach, in relevant part, that human recombinant sRAGE behaves homologously to rat recombinant sRAGE. Specifically, Renard, et al. teach that human recombinant sRAGE (termed "rH-RAGE"), like rat recombinant sRAGE (termed "rR-RAGE"), inhibits AGE-induced endothelial alteration and reduces the hyperpermeability observed in streptozotocin-induced diabetic rat

(see, e.g., abstract and paragraph beginning on the last line of page 1458). Accordingly, applicants maintain that one skilled in the art would reasonably expect that human sRAGE and rat sRAGE are functionally homologous. From the instant specification, one would also expect mouse sRAGE and rat sRAGE to be functionally homologous. Specifically, at page 31, line 15 through page 34, line 3 of the application, experimental results are set forth detailing how murine sRAGE had a beneficial anti-restenotic effect in fatty Zucker rats. It follows, therefore, that human and mouse sRAGE would be expected to be functionally homologous.

In view of the above, applicants maintain that claims 3-5 and 11-14 are enabled and satisfy the requirements of 35 U.S.C. §112, first paragraph.

Summary

For the reasons set forth hereinabove, applicants maintain that the claims pending are in condition for allowance, and respectfully request allowance.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorneys invite the Examiner to telephone them at the number provided below.

No fee, other than the $510.00 fee for a three-month extension of time and the $395.00 RCE fee, is deemed necessary in connection with the filing of this Amendment. However, if any additional fee is required, authorization is hereby given to charge the amount of

such fee to Deposit Account No. 03-3125.

Respectfully submitted,

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The Human and Rat Recombinant Receptors for Advanced Glycation End Products Have a High Degree of Homology but Different Pharmacokinetic Properties in Rats[1]

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ABSTRACT

The accelerated formation of advanced glycation end products (AGEs) is implicated in diabetic microvascular and macrovascular complications. The binding of AGEs to their cellular surface receptor (RAGE) induces vascular dysfunction and in particular an increase in vascular permeability. We previously demonstrated that rat recombinant RAGE (rR-RAGE) produced in insect cells corrected the hyperpermeability due to RAGE-AGE interaction and that pharmacokinetic properties of rR-RAGE after i.v. administration in rats were compatible with a potential therapeutic use. In the present study, we showed that recombinant human RAGE (rH-RAGE) had a similar efficacy in inhibiting AGE-induced endothelial alteration and in reducing the hyperpermeability observed in streptozotocin-induced diabetic rats. ^{125}I-rH-RAGE elimination half-life after i.v. administration was similar in diabetic and normal rats (53.7 ± 7.6 and 45.3 ± 4.0 h, respectively). The presence of AGEs is responsible for a higher distribution volume in diabetic rats compared with normal rats (15.3 ± 2.7 and 7.7 ± 0.7 l/kg, respectively). Immunoreactive ^{125}I-rH-RAGE decreased more rapidly than did immunoreactive ^{125}I-rR-RAGE. The differences between ^{125}I-rH-RAGE and ^{125}I-rR-RAGE pharmacokinetics in rat may be related to differences in potential O-glycosylation and protease cleavage sites between the two RAGE molecules.

Advanced glycation endproducts (AGEs) are a heterogeneous class of molecules found in plasma, cells, and tissues. They accumulate in the vessel wall and the kidney during aging and at an accelerated rate in diabetes (Brownlee, 1995). AGEs are formed by nonenzymatic glycation of primary amino groups on proteins or lipids. The best-characterized AGE receptor (RAGE) (Schmidt et al., 1999) has been purified and cloned in insect cells. RAGE is a member of the Ig superfamily. It is composed of an extracellular region with one V-type domain and two C-type domains, a single transmembrane domain, and a highly charged intracellular domain (Neeper et al., 1992; Schmidt et al., 1994). RAGE is present in various species, and the molecules have a high degree of homology (Neeper et al., 1992; Schmidt et al., 1992). It is expressed on several cell types: endothelial cells (ECs), smooth muscle cells, monocytes/macrophages, cardiac myocytes, neural tissue, and hepatocytes (Brett et al., 1993; Schmidt et al., 1993).

RAGE-AGEs interactions are thought to contribute to the development of diabetic complications, including vascular dysfunction (Wautier et al., 1996; Bierhaus et al., 1997). Our previous studies demonstrated that antibodies directed against RAGE and soluble RAGE purified from bovine lung, a truncated form of the receptor, inhibited the binding of diabetic erythrocytes bearing AGEs to ECs (Wautier et al., 1994). Rat-soluble RAGE or recombinant rat RAGE (rR-RAGE) produced in insect cells reduced the early vascular hyperpermeability observed in diabetic rats (Wautier et al., 1996; Renard et al., 1997). After i.v. administration, RAGE elimination half-life indicated that daily administration was possible (Renard et al., 1997). In fact, in diabetic apolipoprotein E-null mice, i.p. administration of murine-soluble RAGE (40 μg/day for 6 weeks) suppressed accelerated atherosclerosis (Park et al., 1998).

In the present study, we show that recombinant human

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ABBREVIATIONS: AGE, advanced glycation end products; RAGE, receptor for AGEs; rH-RAGE, recombinant human RAGE; EC, endothelial cell; rR-RAGE, recombinant rat RAGE; VCAM-1, vascular cell adhesion molecule 1; PAGE, polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; Fab, fragment having antigen-binding site; RBC, red blood cell; TBIR, tissue-blood isotope ratio; λ_z, terminal disposition rate constant; $T_{1/2}\lambda_z$, elimination half-life; AUC, area under the curve; CL, total clearance; V_z, volume of distribution of the elimination phase; V_c, volume of central compartment; V_{ex}, extrapolated volume; V_{ss}, distribution volume at steady state; MRT, mean residence time; C_{max}, maximal concentration; T_{max}, time of C_{max}.

RAGE (rH-RAGE) is as efficient as rR-RAGE in preventing the increased ^{125}I-albumin transfer through a bovine aortic EC monolayer and in correcting the hyperpermeability observed in diabetic rats. We also compared the pharmacokinetics of ^{125}I-rR-RAGE and ^{125}I-rH-RAGE.

Materials and Methods

Cloning, Expression, and Purification of rH-RAGE and Vascular Cell Adhesion Molecule 1 (VCAM-1)

Human full-length RAGE cDNA was cloned by screening a lung cDNA library (Neeper et al., 1992). Baculovirus expression of rH-RAGE in *Spodoptera frugiperda* (Sf9) cells was obtained as described for rR-RAGE (Renard et al., 1997). The resulting protein obtained corresponded to that expected for the extracellular domain (Schmidt et al., 1992) and migrated as a homogenous sample of ~35 kDa on SDS-polyacrylamide gel electrophoresis (PAGE).

A DNA fragment coding for the first three Ig-like domains of rat VCAM-1 was obtained from a lung cDNA library (Clontech, Palo Alto, CA) by using a polymerase chain reaction technique. The primers used were 5'-GAAATGCCTGTGAAGATGGTCG-3' and 5'-CTCATTGAACAACTAATTCCACTTC-3', based on the known sequence of mouse VCAM-1. A polymerase chain reaction fragment was subcloned into a pCRII vector (Invitrogen, San Diego, CA) for DNA sequencing by the dideoxynucleotide chain-termination method. The deduced amino acid sequence of rat-soluble VCAM-1 has 85% identity with that of murine-soluble VCAM-1. An *EcoRI* fragment of the resultant plasmid was then cloned into a pBacPAK8 vector (Clontech) for the baculovirus expression. The expression and the purification of recombinant-soluble VCAM-1 were essentially the same as for rR-RAGE (Renard et al., 1997) except a Q-Sepharose fast-flow column (Amersham Pharmacia Biotech, Uppsala, Sweden) was used with 20 mM Tris, pH 8.0. The purified recombinant rat soluble VCAM-1 has a molecular mass of around 35 kDa and cross-reacts with antibody against human VCAM-1 (data not shown). The N-terminal sequence of the purified protein is Phe-Lys-Ile-Glu-Ile-Ser-Pro-Glu-Tyr-Lys-Thr-Leu-Ala-Gln-Ile, and it matches with the sequence predicted from that of cDNA.

Database Search

Amino acid sequences of human, rat, and bovine RAGE are available on the Swiss-Prot database. They can be obtained on the World Wide Web (www.expasy.ch). Alignments of the amino acid sequences of human, rat, and bovine RAGE were performed with the SIM program and comparison matrix BLOSUM 62 (Swiss-Prot database). *O*-glycosylation sites were predicted by using the NetOGlyc server (www.cbs.dtu.dk/services/NetOGlyc) (Hansen et al., 1997, 1998).

Radiolabeling of Proteins

rH-RAGE, albumin, and murine Fab Ig fragment were labeled with Na^{125}I by the Iodo-Gen method (Fraker and Speck, 1978; Renard et al., 1997). Precipitation of the iodinated protein by trichloroacetic acid (TCA; 10%) at +4°C was used to determine protein concentrations. Specific activities were in the range of 1 μCi/μg for rH-RAGE, 2.5 μCi/μg for albumin, and 1.15 μCi/μg for murine Fab. Analysis of ^{125}I-rH-RAGE, ^{125}I-albumin, and ^{125}I-Fab preparations by SDS-PAGE and autoradiography indicated no major contamination by label or degradation products for any of the proteins.

In Vitro Permeability Assay

In accordance with provisions of the Declaration of Helsinki and with the rules of our institution, human red blood cells (RBCs) used in our experiments came from normal volunteers and diabetic patients. In vitro permeability assays were performed as previously described (Wautier et al., 1996). ECs were incubated with medium alone or with normal or diabetic RBCs (2.5 × 10^{8} cells/ml) for 24 h. To determine the effect of rH-RAGE on EC permeability, rH-RAGE (60 μg/ml) was added to diabetic RBCs. VCAM-1 (60 μg/ml) was tested in the same experimental conditions as rH-RAGE. To study the permeability, ^{125}I-albumin was added to the upper chamber and the permeability coefficient (P) was determined by the following equation: $P = J \times 1/A \times 1/(C_T - C_B)$, where J is the flux of molecules across the filter; A is the surface area; and C is the concentration of tracer in the top (C_T) and bottom (C_B) chambers (Albelda et al., 1988; Wautier et al., 1996).

In Vivo Permeability Studies

In vivo permeability studies were carried out as previously described (Wautier et al., 1996) in normal and streptozotocin-induced diabetic male Wistar rats. Streptozotocin (45 mg/kg i.v.; Sigma-Aldrich Chimie, Saint-Quentin Fallavier, France) was injected 7 to 9 weeks before experiments. The blood glucose levels of normal and diabetic rats were 90 to 130 mg/dl and above 250 mg/dl, respectively. Normal rats received an i.v. bolus of normal RBCs, diabetic RBCs, diabetic RBCs plus rH-RAGE (5.15 mg/kg), or diabetic RBCs plus VCAM-1 (5.15 mg/kg), and diabetic rats received the same dose of rH-RAGE or VCAM-1 also as an i.v. bolus. In vivo permeability in normal and diabetic rats was determined by using the tissue-blood isotope ratio (TBIR) method (Williamson et al., 1987; Wautier et al., 1996).

Pharmacokinetics of ^{125}I-rH-RAGE and Murine ^{125}I-Fab

Plasma kinetics of ^{125}I-rH-RAGE (250 μg/kg, volume ≤ 4 ml/kg) were studied in normal and streptozotocin-induced diabetic male Wistar rats (200–250 g; CERJ, Laval, France) after i.v. and s.c. administration. Plasma concentrations of ^{125}I-rH-RAGE were determined after precipitation with TCA and also after immunoprecipitation with a specific monoclonal antibody (antibody 9D9). Animals had free access to food and water before the experiments. They were anesthetized with ether before i.v. injection, whereas s.c. injections were given to conscious animals. After the injection, rats were placed in metabolic cages for urine and feces collection. ^{125}I-rH-RAGE was administered by i.v. bolus via the femoral vein. Blood samples (50 μl) were collected in heparin-containing tubes from the tail vein at 2, 5, 10, 30, and 45 min and 1, 1.5, 2, 3, 6, 8, 24, 30, 48, 54, 72, and 96 h and centrifuged for 10 min at 3000g, and plasma was isolated. After s.c. administration, blood samples were collected at the same times except that the first sample was taken at 10 min and additional blood samples were collected at 144, 168, 192, and 216 h. The blood hematocrit measured at 6, 24, 54, and 96 h after ^{125}I-rH-RAGE injection did not differ from physiological values (46%; Davies and Morris, 1993).

After i.v. administration of ^{125}I-rH-RAGE, rats were decapitated at 96 h and radioactivity in organs (intestine, skin, kidney, vena cava, aorta, liver, spleen, lung, heart, and thyroid) was determined. Furthermore, tissue distribution of ^{125}I-rH-RAGE was studied in normal and diabetic rats at the time corresponding to 87.5% of the ^{125}I-rH-RAGE distribution (i.e., 2 and 7.5 h for normal and diabetic rats, respectively). The amount of ^{125}I-rH-RAGE determined in organs was corrected for the presence of radioactivity in the residual blood remaining in the tissue (Ebling et al., 1994).

The pharmacokinetics of a molecule belonging to the Ig superfamily, a monoclonal murine ^{125}I-Fab (250 μg/kg), were also studied in normal rats ($n = 4$) to verify whether the pharmacokinetic profile determined after s.c. injection was influenced by the rH-RAGE characteristics or by the route of injection. The experimental protocol was the same as that for ^{125}I-rH-RAGE.

Identification of ^{125}I-rH-RAGE

Immunoprecipitation of ^{125}I-rH-RAGE. A monoclonal anti-rH-RAGE antibody (antibody 9D9) (Brett et al., 1993) was used in an immunoprecipitation assay to determine the immunoreactive fraction of ^{125}I-rH-RAGE. Samples consisting of ^{125}I-rH-RAGE before

injection diluted in RAGE-free plasma and rat plasma samples taken after injection of ^{125}I-rH-RAGE were analyzed. Samples (50 μl) were incubated with the anti-RAGE antibodies (100 μl), PBS (200 μl), and RAGE-free rat plasma (50 μl) for 1 h at 37°C and overnight at +4°C. A solution of 14% polyethylene glycol 8000 in borate buffer (500 μl; Sigma-Aldrich Chimie) was added, and the mix was incubated overnight. The precipitate obtained by centrifugation (15 min at +4°C and 1800g) was counted in a gamma counter (Packard Instruments).

SDS-PAGE Analysis. After i.v. administration of ^{125}I-rH-RAGE, plasma samples were analyzed by SDS-PAGE. Equal amounts of TCA-precipitable radioactive material (approximately 7000 cpm) from plasma samples were loaded onto a 15% acrylamide gel and analyzed under nonreducing conditions. Radiolabeled proteins and metabolites were autoradiographed with X-ray film (Amersham Pharmacia Biotech) and intensifying screens for 8 weeks. The migration zones of labeled proteins or metabolites were compared with prestained standards (myosin, 202 kDa; β-galactosidase, 137 kDa; BSA, 42.3 kDa; soybean trypsin inhibitor, 31.6 kDa; lysozyme, 18 kDa; aprotinin, 7.6 kDa) (Bio-Rad, Paris, France).

Plasma degradation of rH-RAGE and rR-RAGE was studied in vitro. ^{125}I-rH-RAGE and ^{125}I-rR-RAGE (0.28 μCi/ml) were incubated at 37°C in normal human or rat plasma. An aliquot was taken before incubation and at 1, 3, 6, and 24 h. Samples were immediately frozen at −20°C until analysis by SDS-PAGE and autoradiography for 1 month.

Pharmacokinetic Analysis

Plasma concentration-time data of ^{125}I-rH-RAGE administered i.v. were analyzed by using the Siphar Software (SIMED, Créteil, France) with a noncompartmental method. The terminal disposition rate constant (λ_z) was determined by linear regression analysis and the corresponding half-life ($T_{1/2}\lambda_z$) was calculated as $0.693/\lambda_z$. The area under the plasma ^{125}I-rH-RAGE concentration-time curve from zero to infinity (AUC) was determined by linear trapezoidal estimation from 0 to the last measured time, with extrapolation to infinity by adding the value of the last measured plasma concentration divided by the terminal rate constant (Gibaldi and Perrier, 1982). Total body clearance (CL), distribution volume of the terminal elimination half-life (V_z), extrapolated distribution volume (V_c), and mean residence time (MRT) were calculated by using standard equations (Gibaldi and Perrier, 1982).

Subcutaneous plasma pharmacokinetics of ^{125}I-rH-RAGE and ^{125}I-Fab were also analyzed by a noncompartmental approach to determine $T_{1/2}\lambda_z$ and AUC. The maximal concentration (C_{max}) and the corresponding experimental time (T_{max}) after s.c. administration of ^{125}I-rH-RAGE are the experimental observed values.

Statistical Analysis

Results are presented as mean ± S.E.. One-way ANOVA followed by the parametric Dunnett's test in the event of significant differences was used to compare permeability of ECs in the presence of normal or diabetic RBCs and to analyze the results of in vivo permeability studies. Mean values of pharmacokinetic parameters were compared with the nonparametric Mann-Whitney two-sample test.

Results

In Vitro Permeability

Transfer of ^{125}I-albumin through the EC barrier was similar in culture medium alone and in the presence of normal RBCs. Addition of diabetic RBCs to the medium significantly increased (2.0-fold, $P \leq .001$) the permeability to ^{125}I-albumin. Preincubation of RBCs with rH-RAGE prevented the effect of diabetic RBCs on permeability of the EC monolayer. This effect was dependent on the rH-RAGE concentration, as the permeability was decreased 1.6- and 2.0-fold at rH-RAGE concentrations of 30 and 60 μg/ml, respectively (Fig. 1). VCAM-1 preincubation with diabetic RBCs did not significantly alter the increased passage of ^{125}I-albumin through the EC monolayer.

In Vivo Permeability

In diabetic rats the vascular permeability to ^{125}I-albumin was increased in diabetic compared with normal rats, especially in skin (×2.75), intestine (×2.6), kidney (×2.36), vena cava (×2.2), and heart (×2.1). After a bolus injection of rH-RAGE (5.15 mg/kg) in diabetic rats, the hyperpermeability was corrected. This effect was observed 1 h and 40 min after rH-RAGE injection and was more pronounced in skin and intestine (Fig. 2A). Infusion of diabetic RBCs in normal rats increased the permeability to ^{125}I-albumin compared with normal RBCs infused in normal rats (Fig. 2B). The permeability increase was similar to that observed in diabetic and normal rats infused with rH-RAGE. After diabetic RBC infusion, the vascular permeability to ^{125}I-albumin was 2.5, 2.2, 2.2, 2.1, and 2.0-fold higher in heart, skin, kidney, vena cava, and intestine, respectively. Coadministration of rH-RAGE and diabetic RBCs inhibited the hyperpermeability to ^{125}I-albumin in each organ and particularly in skin (Fig. 2B). VCAM-1 did not reduce the hyperpermeability in organs, indicating that the effect of rH-RAGE was specific.

Plasma Pharmacokinetics after i.v. Administration

Pharmacokinetics of ^{125}I-rH-RAGE Proteins Precipitated by TCA. After i.v. bolus (Fig. 3), ^{125}I-rH-RAGE plasma concentrations decreased more rapidly in diabetic rats than in normal rats and resulted in an AUC 1.4-fold higher in normal than in diabetic rats and in a clearance 1.7-fold higher in diabetic than in normal rats. Distribution clearances (CL_{D1} and CL_{D2}, Table 1) determined by using a three-compartment model (Veng-Pedersen and Gillespie, 1986) for plasma concentration-time curve analysis were not significantly different in normal and diabetic rats, indicating that differences observed in clearance of normal and diabetic

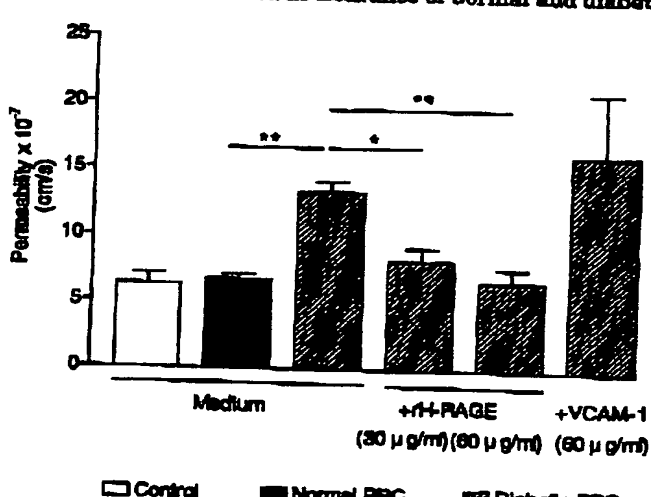

Fig. 1. Effect of diabetic RBCs on the permeability of cultured EC monolayers to ^{125}I-albumin. Monolayers of ECs were incubated with M 199 medium ($n = 7$), with M 199 medium plus normal RBCs ($n = 10$), or with diabetic RBCs ($n = 9$). Effect of rH-RAGE was studied in medium plus diabetic RBCs and rH-RAGE ($n = 5$). The results are presented as mean values. Bars, mean ± S.E.. $^{*}P < .05$; $^{**}P < .01$; and $^{***}P < .001$.

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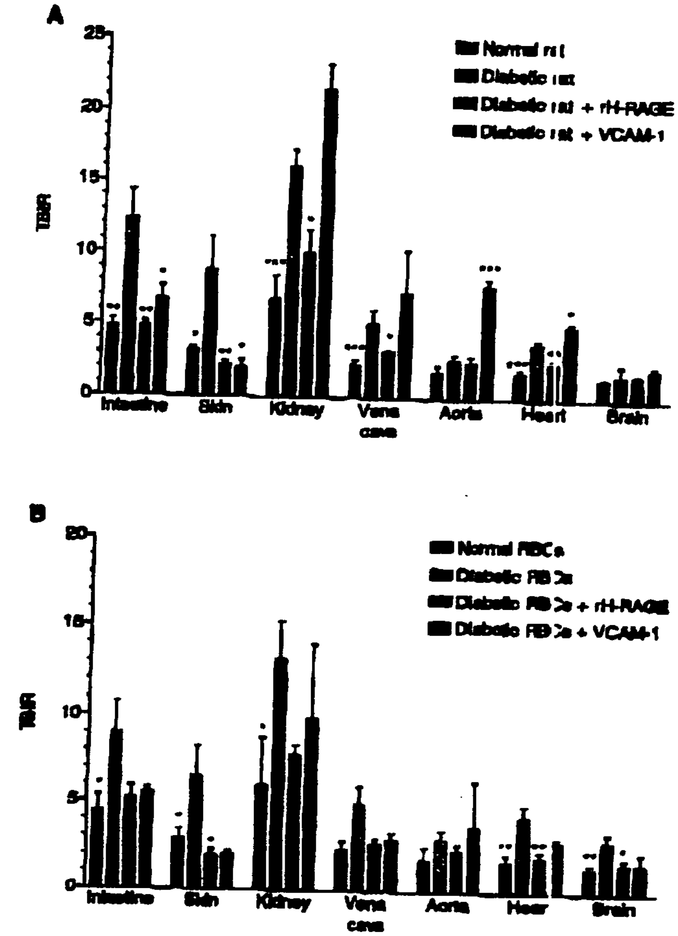

Fig. 2. *A*, effect of *r*H-RAGE on vascular permeability. The TBIR was determined in normal ($n = 6$), diabetic ($n = 7$), and diabetic rats pretreated with *r*H-RAGE (5.15 mg/kg) ($n = 6$). ^{125}I-albumin and ^{51}Cr-RBCs were injected into rats 40 min after RBCs or RBCs plus *r*H-RAGE, and TBIR was calculated according to the formula [^{125}I/^{51}Cr (tissue)] [^{125}I/^{51}Cr (blood)]$^{-1}$, where ^{125}I/^{51}Cr is the ratio of radioactivity in tissue to that in an arterial blood sample harvested before excising the heart. *B*, effect of a transfusion of diabetic RBCs on vascular permeability. Normal RBCs ($n = 7$), diabetic RBCs ($n = 7$), or diabetic RBCs plus *r*H-RAGE (60 μg/ml; $n = 6$) were injected into normal rats and TBIR was determined 1 h after the injection. The results are presented as mean values. Bars, mean ± S.E. $^{*}P < .05$; $^{**}P < .01$; and $^{***}P < .001$.

rats were not due to a different distribution process. The elimination half-life was not significantly different in diabetic and normal rats. The distribution volume was 2-fold higher in diabetic than in normal rats, which is particularly high for a 35-kDa protein, because the total body water of rats is 0.7 l/kg (Davies and Morris, 1993). Because V_z could be influenced by differences in clearance (Jusko and Gibaldi, 1972), distribution volume at steady state (V_{ss}) and extrapolated volume (V_0) were also determined by using a three-compartment model for plasma concentration-time curve analysis (Table 1). Results confirmed the high distribution volume of ^{125}I-rH-RAGE in rat. Volume of the central compartment (V_c) of ^{125}I-rH-RAGE being similar in normal and diabetic rats (Table 1), the difference in the distribution volume was not due to a different ^{125}I-rH-RAGE central compartment in normal and diabetic rats. V_c was approxi-

TABLE 1
Pharmacokinetic parameters of ^{125}I-rH-RAGE precipitated by TCA after i.v. administration in normal and diabetic rats

Rats	Noncompartmental model						Three-Compartment Model				
	$T_{1/2}\lambda_z$	CL	V_z	V_0	MRT	AUC	V_{ss}	V_c	V_t	Cl_{D2}	Cl_{D3}
	h	*ml/h/kg*	*liter/kg*	*liter/kg*	*h*	*ng · h/ml*	*liter/kg*	*liter/kg*	*liter/kg*	*ml/h/kg*	*ml/h/kg*
Diabetic ($n = 5$)	58.7 ± 7.5	208.4 ± 27.6	16.3 ± 2.7	24.1 ± 3.4	51.4 ± 7.8	1342 ± 208	15.2 ± 2.1	86.6 ± 8.2	0.11 ± 0.01	449.3 ± 40.9	285.8 ± 163.9
Normal ($n = 5$)	46.3 ± 4.0	117.4 ± 2.5	7.7 ± 0.7	8.9 ± 1	52.5 ± 2.6	2181 ± 47	8.4 ± 0.8	8.5 ± 1.1	0.11 ± 0.04	639.8 ± 240.6	297.9 ± 205.6
P		<.05	<.05	<.01		<.01	<.01	<.01			

Values are presented as mean ± S.E. $T_{1/2}\lambda_z$, elimination half-life.

mately 2-fold higher than the blood volume (56–86 ml/kg in normal and diabetic rats) and indicates that ^{125}I-rH-RAGE was easily distributed out of the vascular compartment. Because AGEs are extensively formed in diabetic animals they could create an additional distribution compartment, induce a distribution of the protein out of the plasma compartment, and consequently explain the larger distribution volume of the protein in diabetic rats.

Pharmacokinetic parameters of ^{125}I-rH-RAGE are different from those previously obtained for ^{125}I-rR-RAGE (Renard et al., 1997). After i.v. injection of ^{125}I-rR-RAGE, we observed a different elimination half-life in diabetic and normal rats but not with ^{125}I-rH-RAGE. In diabetic rats, ^{125}I-rR-RAGE and ^{125}I-rH-RAGE had a similar elimination half-life (53.74 ± 7.58 and 57.17 ± 11.62 h, respectively, $P = .740$), but in normal rats ^{125}I-rH-RAGE had a higher elimination half-life than did ^{125}I-rR-RAGE (45.27 ± 4.02 and 26.02 ± 2.36 h, respectively, $P = .044$). Distribution volume and total clearance were higher for ^{125}I-rH-RAGE than for ^{125}I-rR-RAGE as shown by a 2.2- and 2.4-fold increase of V_z in diabetic and normal rats and by a 1.9- and 1.3-fold increase of CL in diabetic and normal rats, respectively. AGE concentrations, which are higher in diabetic than in normal rats, might account for the degradation of free ^{125}I-rH-RAGE.

Immunoprecipitation of ^{125}I-rH-RAGE. To further demonstrate that the plasma radioactivity corresponded to intact rH-RAGE, we performed immunoprecipitation studies. Before ^{125}I-rH-RAGE administration in rats, more than 95% of the radioactivity was recovered after TCA precipitation, and 66.2 ± 1.5% was recovered after immunoprecipitation with a monoclonal antibody directed against rH-RAGE. One hour after the i.v. administration, immunoprecipitated ^{125}I-rH-RAGE represented only 46.7 ± 4.7 and 73.0 ± 1.7% of the TCA-precipitable radioactivity in normal and diabetic rats, respectively. Two hours after the administration, immunoprecipitated percentages of the TCA-precipitable radioactivity decreased to 20.7 ± 2.9 and 46.7 ± 0.9%, for normal and diabetic rats, respectively. These results suggest that the monoclonal antibody used in our study recognized ^{125}I-rH-RAGE before its administration in rats, and that after its administration ^{125}I-rH-RAGE was extensively metabolized, especially in normal rats.

SDS-PAGE. This extensive metabolism of ^{125}I-rH-RAGE was also observed by SDS-PAGE (Fig. 4). One hour after

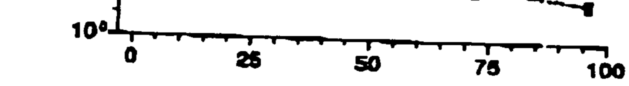

Fig. 3. Plasma concentration versus time profile of ^{125}I-rH-RAGE (250 μg/kg) administered by i.v. route and measured after TCA precipitation in normal ($n = 5$) and diabetic ($n = 5$) rats.

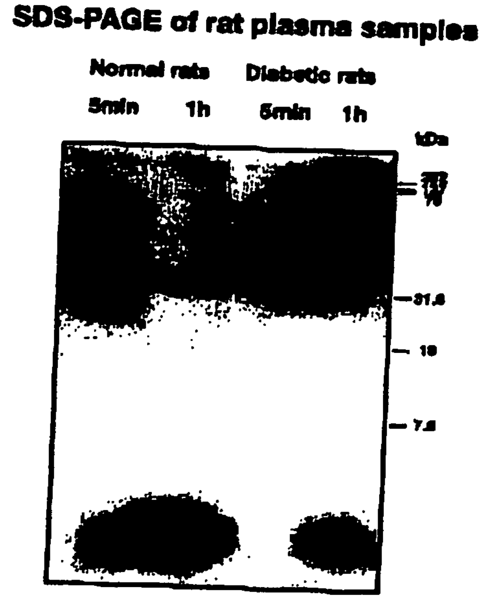

Fig. 4. SDS-PAGE of plasma samples after i.v. administration of ^{125}I-rH-RAGE. Right, standard protein molecular mass (kDa).

^{125}I-rH-RAGE administration in normal and diabetic rats, autoradiography of plasma samples showed one band of approximately 35 kDa and another band of lower molecular mass in normal and diabetic rats. The 35-kDa band could correspond to native ^{125}I-rH-RAGE and the other to ^{125}I-rH-RAGE metabolites. In addition, in plasma samples of diabetic rats, we also observed a third band corresponding to products of higher molecular mass that could correspond to complexes formed with ^{125}I-rH-RAGE. Identification of ^{125}I-rH-RAGE by SDS-PAGE indicated a rapid metabolism of ^{125}I-rH-RAGE, as did the immunoprecipitation studies.

To detect a possible difference in susceptibility of rR-RAGE and rH-RAGE to plasma proteases, we incubated the radiolabeled proteins in rat plasma and human plasma. After a 1-, 3-, 6-, and 24-h incubation in rat plasma, SDS-PAGE and autoradiography showed that the bands corresponding to native rR-RAGE and rH-RAGE (35 kDa) were reduced to a similar extent, as were the bands corresponding to RAGE degradation products. A slower but not significant cleavage of human RAGE was observed in human plasma compared with rat plasma (results not shown).

Plasma Pharmacokinetics after s.c. Administration

Pharmacokinetics of ^{125}I-rH-RAGE Proteins Precipitated by TCA. After s.c. administration, we observed similar elimination half-lives in diabetic and normal rats ($T_{1/2}\lambda_z$ = 64.9 ± 8.5 and 57.9 ± 3.9, respectively, $P = .499$). Two C_{max} values characterized the profile of the ^{125}I-rH-RAGE plasma concentration-time curve. The first around 2 h after the injection and the second 40 to 50 h after the injection (Fig. 5).

Immunoprecipitation of ^{125}I-rH-RAGE. To better understand the two absorption peaks that characterized the s.c. pharmacokinetics of ^{125}I-rH-RAGE, we immunoprecipitated plasma samples at the two C_{max} times (1.8 and 50 h) after s.c. administration of ^{125}I-rH-RAGE in normal rats. Only 16.1 ± 0.6 and 2.3 ± 1.3% of the TCA-precipitable radioactivity were

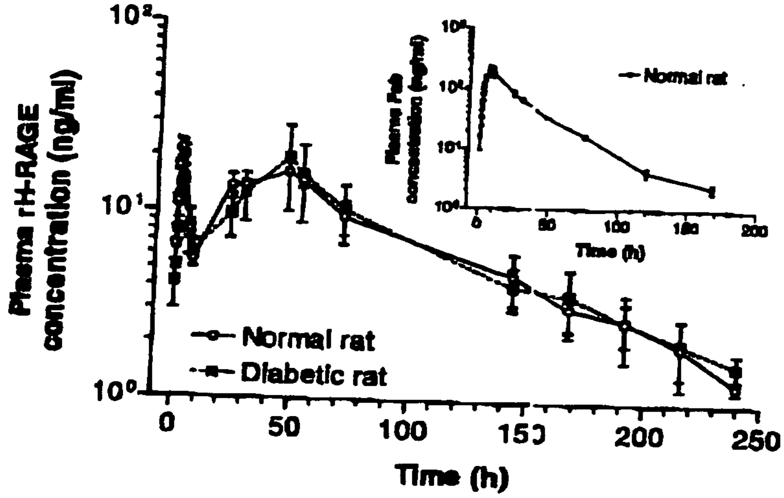

Fig. 5. Plasma concentration versus time profile of ^{125}I-rH-RAGE (250 μg/kg) administered by the s.c. route and measured after TCA precipitation in normal rats ($n = 4$) and diabetic rats ($n = 4$) and of ^{125}I-Fab (250 μg/kg, $n = 4$) administered by the s.c. route and measured after TCA precipitation in normal rats (inset).

immunoprecipitated at 1.8 and 50 h, respectively. In normal rats, ^{125}I-Fab, which was used as a control, was characterized by only one absorption peak, indicating that the mode of administration was not responsible for the two observed C_{max} values with ^{125}I-rH-RAGE. These results could indicate that ^{125}I-rH-RAGE was extensively metabolized after s.c. administration and that the second C_{max} could result from the distribution of radiolabeled metabolites not recognized by the monoclonal antibody used. Although this pharmacokinetic profile is surprising, metabolism of proteins after s.c. injection is well known and has been described for other proteins such as platelet-derived growth factor (PDGF; Abdiu et al., 1998), insulin (Okumura et al., 1985), and parathyroid hormone and calcitonin (Parsons et al., 1979).

Biodistribution of ^{125}I-rH-RAGE

We studied the distribution of ^{125}I-rH-RAGE at the end of the distribution phase after i.v. administration in diabetic ($n = 3$) and normal rats ($n = 3$) (Fig. 6A). The distribution profile of ^{125}I-rH-RAGE in all organs tested was similar in both types of rat. In normal rats, its distribution was 24.3 ± 5.9, 22.0 ± 3.6, 15.3 ± 1.3, and 12.0 ± 3.4% in skin, aorta, kidney, and vena cava, respectively, whereas these percentages were 19.2 ± 3.4, 15.8 ± 2.0, 11.72 ± 3.3, 11.5 ± 3.0, and 10.7 ± 0.8% in aorta, skin, lung, vena cava, and kidney of diabetic rats, respectively. In both types of rat, at the end of the experiment (96 h, Fig. 6B) most of the radioactivity was found in the kidney and aorta at the end of the distribution phase, but it was also elevated in the liver, suggesting hepatic trapping of the protein. It has been reported that the tissue distribution of other proteins such as humanized anti-IgE antibody (Fox et al., 1996) and recombinant human interleukin-11 (Takagi et al., 1995) is higher in the liver and kidney. These findings may reflect residual organ blood and possible metabolism and clearance of the studied protein and its metabolites by these organs (Fox et al., 1996).

Database Search Results

Alignment of the amino acid sequences of human, rat, and bovine RAGE indicated 80.8% identity between human and bovine RAGE, 78% identity between human and rat RAGE, and 73.3% identity between rat and bovine RAGE (Fig. 7). Cysteine residues and potential *N*-glycosylation sites are

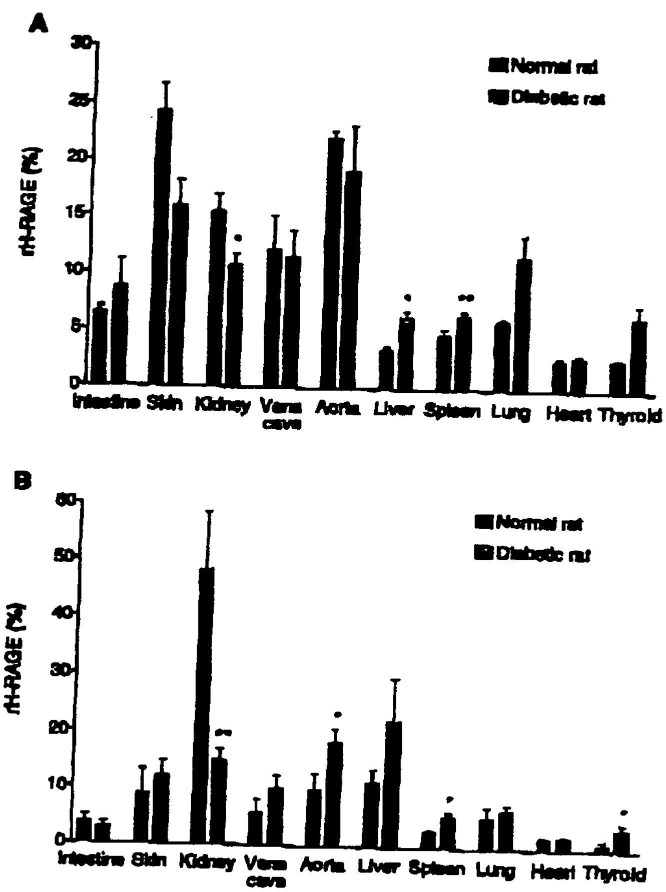

Fig. 6. Biodistributions of radioactivity in normal ($n = 5$) and diabetic ($n = 5$) rats were determined at the end of the experiment (B) and at the end of the distribution phase, i.e., 2 and 7.5 h after the i.v. injection of ^{125}I-rH-RAGE in normal ($n = 3$) and diabetic ($n = 3$) rats, respectively (A). The results are presented as mean percent dose ± S.E. *$P < .05$; **$P < .01$.

conserved in the three molecules. They have several potential *O*-glycosylation sites (T^{204} and S^{207} in human RAGE, T^{194}, S^{280}, S^{305}, and S^{311} in rat RAGE, and T^{101} and S^{317} in bovine

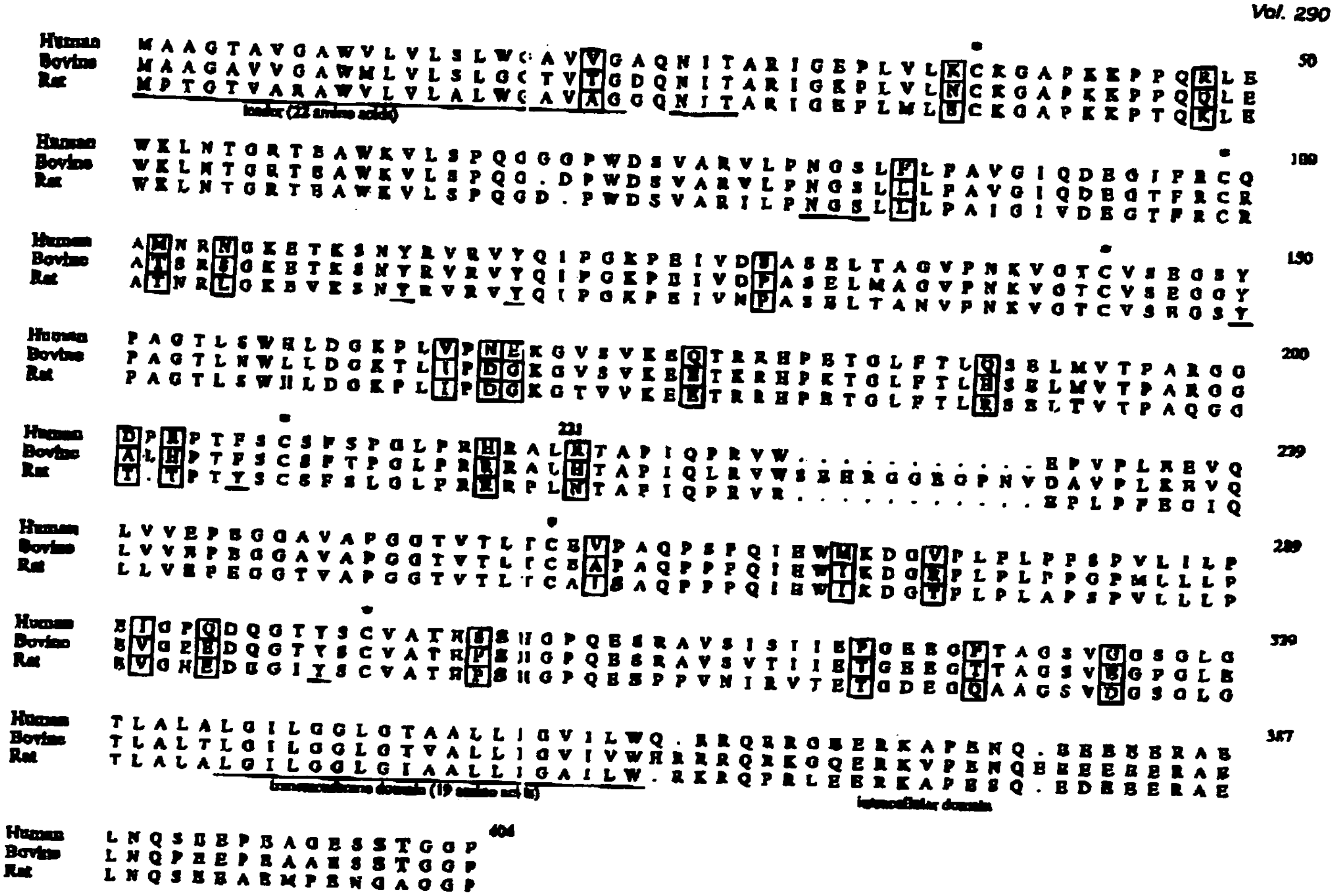

Fig. 7. Alignment of the amino acid sequences of human, bovine (Neeper et al., 1992), and rat (Renard et al., 1997) RAGE. Potential *N*-glycosylation sites are shown by bold underlining, and tyrosine residues (?) are shown by light underlining. Cysteine residues involved in Ig domains are marked by asterisks. Boxed amino acids correspond to residues different among the three RAGE molecules. Residues 31–106, 31–105, and 31–106 represent the Ig-like V domain of human, bovine, and rat RAGE, respectively. Residues 137–214, 136–212, and 136–218 represent the first Ig-like C2 domain of human, bovine, and rat RAGE, respectively. Residues 251–308, 262–318, and 250–306 represent the second Ig-like C2 domain of human, bovine, and rat RAGE, respectively.

RAGE), and only one is conserved in all three molecules (S^{207} of human RAGE, S^{208} of rat RAGE, and S^{217} of bovine RAGE). Several potential sites of proteolysis by trypsin-like enzymes are present in RAGE from different species, but the only major difference is that human RAGE has an arginine (R^{221}), which may be responsible for an increased susceptibility of human RAGE to trypsin-like enzymes, and may affect the half-life of rH-RAGE in rat plasma.

Discussion

After i.v. injection, pharmacokinetic parameters of ^{125}I-rR-RAGE are different in normal and diabetic rats. This finding is probably related to the presence in diabetic animals of AGEs, which create an additional distribution compartment for ^{125}I-rR-RAGE (Renard et al., 1997). After i.v. injection, ^{125}I-rH-RAGE had a higher distribution volume and a higher clearance in diabetic than in normal rats, but the ^{125}I-rH-RAGE elimination half-life was similar. It is unlikely that differences were due to the alteration of proteins during iodination (Bauer et al., 1996), because rR-RAGE and rH-RAGE were radiolabeled by the same protocol and have no major difference in their phenylalanine and tyrosine contents.

Immunoprecipitation by anti-RAGE antibody is more specific than TCA precipitation, and plasma concentrations determined by TCA precipitation might be overestimated, while those determined by immunoprecipitation might be underestimated, since a small conformational change may result in nonreactivity with the monoclonal antibody used. Despite this discrepancy between the two methods, immunoreactive ^{125}I-rH-RAGE plasma concentrations decrease rapidly after i.v. injection, whereas 93% of ^{125}I-rR-RAGE is immunoprecipitable 2 h after i.v. injection (Renard et al., 1997). The low percentage of immunoprecipitated ^{125}I-rH-RAGE in the present study might be due to several factors: ^{125}I-rH-RAGE may be more catabolized by proteases, a different glycosylation between rH-RAGE and rR-RAGE can lead to a different metabolism, or ^{125}I-rH-RAGE may form complexes with AGEs that modify its conformation.

Pharmacokinetic profiles after s.c. injection of ^{125}I-rH-RAGE also suggested a rapid metabolism of the human protein in rat. Human recombinant ^{125}I-tumor necrosis factor α

(TNF-α) has a longer elimination half-life in monkeys than in rabbits (Bocci et al., 1987), which further demonstrates the importance of the experimental model. In our in vitro degradation experiments we did not detect a difference between rR-RAGE and rH-RAGE stability in rat plasma, which might have explained the difference observed in the animal experiments. However, the sensitivity of the technique, even after densitometry analysis, is low. A possible complex mechanism involving cell surface associated proteases or surface components could be responsible for the difference between rR-RAGE and rH-RAGE pharmacokinetics in the rat. In the same rat model, we previously showed that purified bovine ^{125}I-RAGE pharmacokinetics are similar to those of ^{125}I-rR-RAGE (Wautier et al., 1996; Renard et al., 1997), which indicates that differences between human and rat RAGE cannot simply be explained by the heterologous nature of the proteins. To investigate possible explanations, we compared the amino acid sequences of rat, human, and bovine RAGE and found some differences between the three molecules. The arginine-221 in the human protein could be an additional cleavage site for trypsin-like enzymes, resulting in fragments of 24 and 13 kDa that both have tyrosine residues and remain radiolabeled. This could explain the extensive catabolism of ^{125}I-rH-RAGE and the presence of radiolabeled low molecular weight products on SDS-PAGE. Further studies using a mutated rH-RAGE with a substitution of arginine-221 would be useful to test this hypothesis. Prediction of different potential *O*-glycosylation sites suggests an alternative hypothesis, because human, rat, and bovine RAGE have different potential *O*-glycosylation sites that could lead to a different glycosylation pattern. Previous studies on protein pharmacokinetics indicate that carbohydrate moieties modulate catabolism of proteins, as observed with tissue plasminogen activator (Lucore et al., 1988) and interferon β (Bocci et al., 1982).

In vitro and in vivo, rR-RAGE (Renard et al., 1997) and rH-RAGE reverse vascular permeability produced by AGEs. Despite the catabolism of ^{125}I-rH-RAGE in rat, the biological efficacy indicates that rH-RAGE fragments are still active or, as observed in genetically modified mice, that recombinant RAGE is active at a low concentration (Park et al., 1998). Delineation of the active peptide of the rH-RAGE molecule might allow better understanding of the discrepancy between the immunoreactive rH-RAGE blood level and its biological activity. Development of rH-RAGE as a treatment for reversing alterations due to AGEs will require further studies to improve stability and bioavailability of the protein. Protection of rH-RAGE from degradation in the bloodstream or the use of injectable depot formulations, in which rH-RAGE would be embedded in a polymeric matrix or vesicles (e.g., liposomes) (Putney and Burke, 1998) and released slowly, could be methods for overcoming degradation after i.v. or s.c. administration.

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